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<p>(21) International Application Number: PCT/US92/02854</p> <p>(22) International Filing Date: 8 April 1992 (08.04.92)</p> <p>(30) Priority data: 682,812 9 May 1991 (09.05.91) US</p> <p>(60) Parent Application or Grant (63) Related by Continuation US 682,812 (CIP) Filed on 9 May 1991 (09.05.91)</p> <p>(71) Applicant (for all designated States except US): TEMPLE UNIVERSITY OF THE COMMONWEALTH SYSTEM OF HIGHER EDUCATION [US/US]; Philadelphia, PA 19122 (US).</p> <p>(72) Inventors; and (75) Inventors/Applicants (for US only) : GEWIRTZ, Alan, M. [US/US]; 837 North 24th Street, Philadelphia, PA 19130 (US). CALABRETTA, Bruno [IT/US]; 2401 Pine Street, Philadelphia, PA 19103 (US).</p>		<p>(74) Agent: MONACO, Daniel, A.; Seidel, Gonda, Lavorgna & Monaco, Two Penn Center Plaza, Suite 1800, Philadelphia, PA 19102 (US).</p> <p>(81) Designated States: AT, AT (European patent), AU, BB, BE (European patent), BF (OAPI patent), BG, BJ (OAPI patent), BR, CA, CF (OAPI patent), CG (OAPI patent), CH, CH (European patent), CI (OAPI patent), CM (OAPI patent), CS, DE, DE (European patent), DK, DK (European patent), ES, ES (European patent), FI, FR (European patent), GA (OAPI patent), GB, GB (European patent), GN (OAPI patent), GR (European patent), HU, IT (European patent), JP, KP, KR, LK, LU, LU (European patent), MC (European patent), MG, ML (OAPI patent), MN, MR (OAPI patent), MW, NL, NL (European patent), NO, PL, RO, RU, SD, SE, SE (European patent), SN (OAPI patent), TD (OAPI patent), TG (OAPI patent), US.</p> <p>Published <i>With international search report.</i></p>														
<p>(54) Title: ANTISENSE OLIGONUCLEOTIDES TO C-KIT PROTO-ONCOGENE AND USES THEREOF</p> <div data-bbox="511 1234 1039 1654"> <table border="1"> <caption>Approximate data from bar chart</caption> <thead> <tr> <th>Bar Pattern</th> <th>Colonies / 2x10⁴ Cells Plated</th> </tr> </thead> <tbody> <tr> <td>Solid Black</td> <td>~120</td> </tr> <tr> <td>Diagonal (TL-BR)</td> <td>~115</td> </tr> <tr> <td>Diagonal (BL-TL)</td> <td>~85</td> </tr> <tr> <td>Horizontal Lines</td> <td>~45</td> </tr> <tr> <td>Dotted</td> <td>~130</td> </tr> <tr> <td>Vertical Lines</td> <td>~110</td> </tr> </tbody> </table> </div> <p>(57) Abstract</p> <p>Oligonucleotides are provided having a nucleotide sequence complementary to at least a portion of the mRNA transcript of the human <i>c-kit</i> gene. These "antisense" oligonucleotides are hybridizable to the <i>c-kit</i> mRNA transcript. Such oligonucleotides are useful in selectively inhibiting proliferation of erythroid cells, particularly in disorders characterized by an elevated hematocrit due to overproduction of erythrocytes. The antisense oligomers also have activity agent hematologic neoplastic cells and are therefore suitable as bone marrow purging agents.</p>			Bar Pattern	Colonies / 2x10 ⁴ Cells Plated	Solid Black	~120	Diagonal (TL-BR)	~115	Diagonal (BL-TL)	~85	Horizontal Lines	~45	Dotted	~130	Vertical Lines	~110
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ANTISENSE OLIGONUCLEOTIDES TO C-KIT
PROTO-ONCOGENE AND USES THEREOF

Field of the Invention

5 The invention relates to antisense oligonucleotides to proto-oncogenes, in particular to antisense oligonucleotides to the c-kit gene, and the use of such oligonucleotides to selectively inhibit proliferation of certain cells.

10 **Reference to Government Grant**

 The invention described herein was supported in part by National Institutes of Health grants CA36896 and CA01324. The United States government has certain rights
15 in the invention.

Background of the Invention

 The c-kit gene is the normal homologue of v-kit, the HZ4 feline sarcoma virus oncogene. It resides
20 on human chromosome 4. The gene encodes a dimeric transmembrane glycoprotein receptor with tyrosine kinase activity that appears to be highly related to the receptors for colony stimulating factor-1 and platelet derived growth factor. (Yarden et. al., The EMBO Journal, 6, 3341-3351 (1987)). Like these receptors, c-kit also
25 appears to belong to the immunoglobulin gene superfamily.

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The mouse c-kit gene has been mapped to chromosome 5 where it was determined to be allelic with the dominant white spotting locus (W) (Chabot et al., Nature 335, 88-89 (1988). C-kit mutations are commonly found in W mice and, in addition to abnormalities affecting coat color and gonadal development, they also have a variety of hematopoietic defects. Macrocytic anemia is one of the most striking and profound of these abnormalities. The W⁴² mutation, associated with a particularly severe hematologic manifestation, has been shown to be due to a missense mutation leading to replacement of one amino acid and defective tyrosine kinase activity (Tan et al., Science 247, 209 (1990)). Such animals are also known to have about one-third of the erythroid burst forming units of healthy wild-type littermates (Goldwather et al., Exp. Heme. 18, 936 (1990)).

The ligand for the c-kit receptor has now been identified, molecularly cloned and expressed (Yarden et al., The EMBO Journal, 6, 3341-3351 (1987)). The encoded protein, known as stem cell factor (SCF), mast cell growth factor (MGF), or steel factor (SLF) is the product of a gene which resides at the steel (Sl) locus. Mice with Sl mutations have phenotypic abnormalities quite similar to those of W mice. The W mouse lacks, or has defects in, a critical signal transducing receptor encoded by c-kit. The Sl mouse has defects in the ligand which stimulates the receptor.

The importance of the c-kit ligand-receptor system in human hematopoiesis is unclear. No human mutations at the corresponding loci have been described. Studies in mice may have very limited applicability to human systems. Moreover, even if a tissue is shown to express a particular message, the importance of the message to expression of a cellular phenotype is not known until the cell is deprived of the encoded protein. Biological systems are redundant. Lack of a protein can often be compensated by another protein of the same fami-

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ly. It is therefore not predictable that inhibition of expression of a particular gene will result in altered phenotype.

Summary of the Invention

Antisense oligonucleotides and pharmaceutical compositions thereof with pharmaceutical carriers are provided. Each oligonucleotide has a nucleotide sequence complementary to at least a portion of the mRNA transcript of the human c-kit gene. The oligonucleotide is hybridizable to the mRNA transcript. Preferably, the oligonucleotide is at least a 12-mer oligonucleotide, that is, an oligomer containing at least 12 nucleotide residues. In particular, the oligomer is advantageously a 12-mer to a 40-mer, preferably an oligodeoxynucleotide. While oligonucleotides smaller than 12-mers may be utilized, they are statistically more likely to hybridize with non-targeted sequences, and for this reason may be less specific. In addition, a single mismatch may destabilize the hybrid. While oligonucleotides larger than 40-mers may be utilized, uptake may be more difficult. Moreover, partial matching of long sequences may lead to non-specific hybridization, and non-specific effects. Preferably, the oligonucleotide is a 15- to 30-mer oligodeoxynucleotide, more advantageously an 18- to 26-mer. A 15- to 21-mer is most preferred.

While in principle oligonucleotides having a sequence complementary to any region of the c-kit gene find utility in the present invention, oligonucleotides complementary to a portion of the c-kit mRNA transcript including the translation initiation codon are particularly preferred. Also preferred are oligonucleotides complementary to a portion of the c-kit mRNA transcript lying within about 40 nucleotides upstream (the 5' direction) or about 40 nucleotides downstream (the 3' direction) from the translation initiation codon.

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The invention also provides a method for inhibiting proliferation of erythroid cells comprising administering to a host in need of such treatment an effective amount of the c-kit antisense oligonucleotides of the invention.

The invention provides a method for treating hematologic neoplasms characterized by c-kit expression comprising administering an effective amount of c-kit antisense oligonucleotide in vivo or ex vivo to a host in need of such treatment, or to cells harvested from the host.

Administration of the c-kit oligonucleotides is also useful in treatment of malignant melanoma, and testicular or ovarian tumors.

As used in the herein specification and appended claims, unless otherwise indicated, the term "oligonucleotide" includes both oligomers of ribonucleotide i.e., oligoribonucleotides, and oligomers of deoxyribonucleotide i.e., oligodeoxyribonucleotides (also referred to herein as "oligodeoxynucleotides"). Oligodeoxynucleotides are preferred.

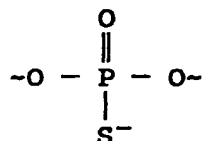
As used herein, unless otherwise indicated, the term "oligonucleotide" also includes oligomers which may be large enough to be termed "polynucleotides".

The terms "oligonucleotide" and "oligodeoxynucleotide" include not only oligomers and polymers of the biologically significant nucleotides, i.e. nucleotides of adenine ("A"), deoxyadenine ("dA"), guanine ("G"), deoxyguanine ("dG"), cytosine ("C"), deoxycytosine ("dC"), thymine ("T") and uracil ("U"), but also oligomers and polymers hybridizable to the c-kit mRNA transcript which may contain other nucleotides. Likewise, the terms "oligonucleotide" and "oligodeoxynucleotide" include oligomers and polymers wherein one or more purine or pyrimidine moieties, sugar moieties or internucleotide linkages is chemically modified. The term "oligonucleotide" is thus understood to also include oligomers which may prop-

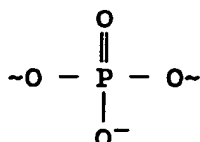
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erly be designated as "oligonucleosides" because of modification of the internucleotide phosphodiester bond. Such modified oligonucleotides include, for example, the alkylphosphonate oligonucleosides, discussed below.

The term "phosphorothioate oligonucleotide" means an oligonucleotide wherein one or more of the internucleotide linkages is a phosphorothioate group,

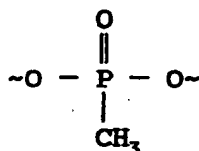


as opposed to the phosphodiester group



which is characteristic of unmodified oligonucleotides.

By "alkylphosphonate oligonucleoside" is meant an oligonucleotide wherein one or more of the internucleotide linkages is an alkylphosphonate group,



wherein R is an alkyl group, preferably methyl or ethyl.

The term "downstream" when used in reference to a direction along a nucleotide sequence means the 5'→3' direction. Similarly, the term "upstream" means the 3'→5' direction.

The term "c-kit mRNA transcript" means the presently known mRNA transcript of the human c-kit gene, or any further transcripts which may be elucidated.

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Description of the Figures

Figure 1 is an autoradiograph of a reverse transcription-polymerase chain reaction gel indicating the increase in c-kit mRNA at intervals following stimulation of adherent, T lymphocyte-depleted human bone marrow cells (A^TMNC) with 20 U/ml interleukin-3 and 5 U/ml erythropoietin: Lane 1 (t = 0); lane 2 (2 hrs); lane 3 (8 hrs); lane 4 (12 hrs); lane 5 (24 hrs); lane 6 (36 hrs); lane 7 (48 hrs); lane 8 (H₂O control).

Figure 2 is a similar autoradiograph indicating the effect of c-kit oligomer exposure on c-kit mRNA levels in A^TMNC after stimulation by interleukin-3 and erythropoietin in 5% AB serum. Lane 1 (no oligomer, t = 0); lane 2 (no oligomer, t = 36 hrs); lane 3 (sense oligomer, 36 hrs); lane 4 (antisense, 36 hrs); lane 5 (scrambled sequence with identical base content, 36 hrs).

Figure 3 shows the effect of c-kit oligodeoxynucleotides on BFU-E-derived colony formation. Oligomers were added to cultures at time zero, and 50% of the initial dose was again added 18 hours later. The bars on the graph indicate: 1, untreated control cells; 2, antisense treatment of 20 µg/ml followed by 10 µg/ml; 3, antisense treatment of 40 µg/ml followed by 20 µg/ml; 4, antisense treatment of 100 µg/ml followed by 50 µg/ml; 5, sense treatment of 100 µg/ml followed by 50 µg/ml; 6, scrambled-sequence treatment of 100 µg/ml followed by 50 µg/ml.

Detailed Description of the Invention

We have discovered that the c-kit gene is of predominant importance in human erythropoiesis. We have found that the protein which this gene expresses, a receptor for tyrosine kinase, transduces a signal which acts in concert with interleukin-3 (IL-3) to optimize cell proliferation, particularly erythroid burst forming units (BFU-E).

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The putative DNA sequence complementary to the mRNA transcript of the human c-kit gene has been reported by Yarden et al., The EMBO Journal, 6, 3341-3351 (1987), the entire disclosure of which is incorporated herein by reference. The nucleotide sequence and predicted amino acid sequence are set forth in Figure 3 thereof. The c-kit polypeptide is synthesized by translation of a single 5-kb mRNA, which contains an open reading frame coding for a 976 amino acid polypeptide.

The antisense oligonucleotides of the invention may be synthesized by any of the known chemical oligonucleotide synthesis methods. Such methods are generally described, for example, in Winnacker, From Genes to Clones: Introduction to Gene Technology, VCH Verlagsgesellschaft mbH (H. Ibelgauf's trans. 1987).

Any of the known methods of oligonucleotide synthesis may be utilized in preparing the instant antisense oligonucleotides.

The antisense oligonucleotides are most advantageously prepared by utilizing any of the commercially available, automated nucleic acid synthesizers, for example, the Applied Biosystems 380B DNA Synthesizer, which utilizes β -cyanoethyl phosphoramidite chemistry.

Since the complete nucleotide synthesis of DNA complementary to the c-kit mRNA transcript is known, antisense oligonucleotides hybridizable with any portion of the mRNA transcript may be prepared by the oligonucleotide synthesis methods known to those skilled in the art.

While any length oligonucleotide may be utilized in the practice of the invention, sequences shorter than 12 nucleotides may be less specific in hybridizing to the target c-kit mRNA, may be more easily destroyed by enzymatic digestion, and may be destabilized by even a single base pair mismatch. Hence, oligo-

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nucleotides having 12 or more nucleotides are preferred.

Long sequences, particularly sequences longer than about 40 nucleotides, may be somewhat less effective in inhibiting c-kit translation because of decreased uptake by the target cell. Thus, oligomers of 12-40 nucleotides are preferred, more preferably 15-30 nucleotides, most preferably 18-26 nucleotides. Sequences of 18-21 nucleotides are particularly preferred. While sequences of 18-21 nucleotides are most particularly preferred, for unmodified oligonucleotides, slightly longer chains of up to about 26 nucleotides, are preferred for modified oligonucleotides such as phosphorothioate oligonucleotides, which hybridize less strongly to mRNA than unmodified oligonucleotides.

Oligonucleotides complementary to and hybridizable with any portion of the c-kit mRNA transcript are, in principle, effective for inhibiting translation of the transcript, and capable of inducing the effects herein described. It is believed that translation is most effectively inhibited by blocking the mRNA at a site at or near the initiation codon. Thus, oligonucleotides complementary to the 5'-terminal region of the c-kit mRNA transcript are preferred. The oligonucleotide is preferably directed to a site at or near the initiation codon for protein synthesis. The following 40-mer oligodeoxynucleotide is complementary to the c-kit mRNA transcript beginning with the initiation codon of the transcript and extending downstream (in the 5' direction):

GAACGCAGAG AAAATCCCAG GCGCCGCGAG
CGCCTCTCAT (SEQ ID NO:1).

Smaller oligomers based upon the above sequence, in particular oligomers hybridizable to segments of the c-kit message containing the initiation codon, may be utilized. Particularly preferred are the following 15- to 26-mers:

(SEQ ID NO:2)

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(SEQ ID NO:3)

(SEQ ID NO:4)

(SEQ ID NO:5)

(SEQ ID NO:6)

5

(SEQ ID NO:7)

(SEQ ID NO:8)

(SEQ ID NO:9)

(SEQ ID NO:10)

(SEQ ID NO:11)

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(SEQ ID NO:12)

(SEQ ID NO:13)

Oligonucleotides hybridizable to the c-kit mRNA transcript finding utility according to the present invention include not only native oligomers of the biologically significant nucleotides, i.e., A, dA, G, dG, C, dC, T and U, but also oligonucleotide species which have been modified for improved stability and/or lipid solubility. The oligonucleotides may be any of a number of types, including those having a charged or uncharged backbone. For example, it is known that enhanced lipid solubility and/or resistance to nuclease digestion results by substituting an alkyl group or sulfur atom for a phosphate oxygen in the internucleotide phosphodiester linkage to form alkylphosphonate oligonucleotide or phosphorothioate oligonucleotides. The phosphorothioates, in particular, are stable to nuclease cleavage and soluble in lipid. They may be synthesized by known automatic synthesis methods.

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The oligonucleotide employed may represent an unmodified oligonucleotide or an oligonucleotide analog. One group of such analogs, the alkyl phosphonates, includes but is not limited to the ethyl or methyl phosphonate analogs disclosed by U.S. Patent No. 4,469,863.

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Non-ionic oligonucleotides are characterized by increased resistance to nuclease hydrolysis and/or increased cellular uptake, while retaining the ability

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to form stable complexes with complementary nucleic acid sequences. The alkylphosphonates in particular, are stable to nuclease cleavage and soluble in lipid. The preparation of alkylphosphonate oligonucleosides is disclosed in U.S. Patent 4,469,863.

Methylphosphonate oligomers can be prepared by a variety of methods, both in solution and on insoluble polymer supports (Agrawal and Riftina, Nucl. Acids Res., 6, 3009-3024 (1979); Miller et al., Biochemistry, 18, 5134-5142 (1979), Miller et al., J. Biol. Chem., 255, 9659-9665 (1980); Miller et al., Nucl. Acids Res., 11, 5189-5204 (1983), Miller et al., Nucl. Acids Res., 11, 6225-6242 (1983), Miller et al., Biochemistry, 25, 5092-5097 (1986); Engels and Jager, Angew. Chem. Suppl. 912 (1982); Sinha et al., Tetrahedron Lett. 24, 877-880 (1983); Dorman et al., Tetrahedron, 40, 95-102 (1984); Jager and Engels, Tetrahedron Lett., 25, 1437-1440 (1984); Noble et al., Nucl. Acids Res., 12, 3387-3404 (1984); Callahan et al., Proc. Natl. Acad. Sci. USA, 83, 1617-1621 (1986); Koziolkiewicz et al., Chemica Scripta, 26, 251-260 (1986); Agrawal and Goodchild, Tetrahedron Lett., 38, 3539-3542 (1987); Lesnikowski et al., Tetrahedron Lett., 28, 5535-5538 (1987); Sarin et al., Proc. Natl. Acad. Sci. USA, 85, 7448-7451 (1988)).

The most efficient procedure for preparation of methylphosphonate oligonucleosides involves use of 5'-O-dimethoxytrityldeoxynucleoside-3'-O-diisopropylmethylphosphoramidite intermediates, which are similar to the methoxy or β -cyanoethyl phosphoramidite reagents used to prepare oligodeoxyribonucleotides. The methylphosphonate oligomers can be prepared on controlled pore glass polymer supports using an automated DNA synthesizer (Sarin et al., Proc. Natl. Acad. Sci. USA, 85, 7448-7451 (1988)).

Resistance to nuclease digestion may also be achieved by modifying the internucleotide linkage at both the 5' and 3' termini with phosphoroamidites ac-

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according to the procedure of Dagle et al., Nucl. Acids Res. 18, 4751-4757 (1990).

Phosphorothioate oligonucleotides contain a sulfur-for-oxygen substitution in the internucleotide phosphodiester bond. Phosphorothioate oligonucleotides combine the properties of effective hybridization for duplex formation with substantial nuclease resistance, while retaining the water solubility of a charged phosphate analogue. The charge is believed to confer the property of cellular uptake via a receptor (Loke et al., Proc. Natl. Acad. Sci. U.S.A. 86, 3474-3478 (1989)).

Phosphorothioate oligodeoxynucleotide are described by LaPlanche, et al., Nucleic Acids Research 14, 9081 (1986) and by Stec et al., J. Am. Chem. Soc. 106, 6077 (1984). The general synthetic method for phosphorothioate oligonucleotides was modified by Stein et al., Nucl. Acids Res., 16, 3209-3221 (1988), so that these compounds may readily be synthesized on an automatic synthesizer using the phosphoramidite approach.

Furthermore, recent advances in the production of oligoribonucleotide analogues mean that other agents may also be used for the purposes described here, e.g., 2'-O-methylribonucleotides (Inoue et al., Nucleic Acids Res. 15, 6131 (1987) and chimeric oligonucleotides that are composite RNA-DNA analogues (Inoue et al., FEBS Lett. 215, 327 (1987)).

While inhibition of c-kit mRNA translation is possible utilizing either antisense oligoribonucleotides or oligodeoxyribonucleotides, free oligoribonucleotides are more susceptible to enzymatic attack by ribonucleases than oligodeoxyribonucleotides. Hence, oligodeoxyribonucleotides are preferred in the practice of the present invention. Oligodeoxyribonucleotides are further preferred because, upon hybridization with c-kit mRNA, the resulting DNA-RNA hybrid duplex is a substrate for RNase H, which specifically attacks the

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RNA portion of DNA-RNA hybrid. Degradation of the mRNA strand of the duplex releases the antisense oligodeoxynucleotide for hybridization with additional c-kit messages.

5 In general, the antisense oligonucleotides of the present invention will have a sequence which is completely complementary to the target portion of the c-kit message. Absolute complementarity is not however required, particularly in larger oligomers. Thus,
10 reference herein to a "nucleotide sequence complementary to at least a portion of the mRNA transcript" of c-kit does not necessarily mean a sequence having 100% complementarity with the transcript. In general, any oligonucleotide having sufficient complementarity to
15 form a stable duplex with c-kit mRNA, that is, an oligonucleotide which is "hybridizable", is suitable. Stable duplex formation depends on the sequence and length of the hybridizing oligonucleotide and the degree of complementarity with the target region of the
20 c-kit message. Generally, the larger the hybridizing oligomer, the more mismatches may be tolerated. More than one mismatch probably will not be tolerated for antisense oligomers of less than about 21 nucleotides. One skilled in the art may readily determine the degree of mismatching which may be tolerated between any
25 given antisense oligomer and the target c-kit message sequence, based upon the melting point, and therefore the stability of the resulting duplex. Melting points of duplexes of a given base pair composition can be
30 determined from standard texts, such as Molecular Cloning: A Laboratory Manual, (2nd edition, 1989), J. Sambrook et al., eds.

35 While oligonucleotides capable of stable hybridization with any region of the c-kit message are within the scope of the present invention, oligonucleotides complementary to a region including the initiation codon are believed particularly effective. Par-

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5 ticularly preferred are oligonucleotides hybridizable to a region of the c-kit mRNA up to 40 nucleotides upstream (in the 5' direction) of the initiation codon or up to 40 nucleotides downstream (in the 3' direction) of that codon.

10 The antisense oligonucleotides of the invention inhibit human erythropoiesis, as indicated by inhibition of colony forming unit-erythroid cells (CFU-E) and burst forming unit-erythroid cells (BFU-E). However, they do not appear to inhibit proliferation of cells of other lineages, such as colony forming unit-granulocyte-macrophage cells (CFU-GM) and colony forming unit-megakaryocyte cells (CFU-MEG). CFU-GM cells and CFU-MEG cells are the progenitors of blood granulocytes and platelets, respectively. This pharmaceuti-
15 cally significant differential sensitivity makes the instant oligonucleotides useful in treating disorders characterized by elevated production of red blood cells.

20 The antisense oligonucleotides of the invention are believed useful in the treatment of any one of a variety of conditions characterized by an elevated hematocrit due to overproduction of erythrocytes. One such disorder, polycythemia, may arise from a variety of causes and is classified as either relative, secondary or primary polycythemia.
25

In relative polycythemia, the red cell mass is normal. Plasma volume is decreased. The increase in erythrocytes is therefore a concentration effect.
30 Relative polycythemia is associated with diabetic acidosis, diarrhea, or diabetes insipidus. It is also associated with the intake of diuretics.

In secondary polycythemia, red cell mass is increased secondarily to elevated erythropoietin (EPO) production. This occurs in individuals who have located to higher altitudes, since decreased oxygen stimulates anemia, which is a triggering signal for increase
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of erythrocyte production. Secondary polycythemia may also occur in patients who have significant pulmonary or cardiac disfunction. Decreased oxygen delivery to tissues simulates anemia which triggers a signal to increase erythrocyte production. Secondary polycythemia may also occur in individuals who have tumors which are capable of synthesizing EPO, as in hypernephroma, cerebellar hemangioma and uterine leiomyoma.

Primary polycythemia is characterized by an increase in red cell mass, with either normal or decreased EPO levels. Primary polycythemia occurs in the myeloproliferative disorders, in particular polycythemia vera (PV). Disorders such as PV are true stem cell disorders. Accordingly, the white blood cell count and platelet count may be elevated. However, control of erythrocyte production is the primary objective in management of PV. Control of PV is usually effected by phlebotomy in secondary causes (if treatment of the primary disease is ineffective), and by a combination of phlebotomy and chemotherapy. Chemotherapeutic treatment of PV typically utilizes alkylating agents such as busulfan, melphalan, cyclophosphamide, chlorambucil or radioactive phosphorous in the form of sodium phosphate-³²P.

The rapid fluid shifts imposed by phlebotomy in the treatment of PV can be dangerous for patients with cardiac/pulmonary disease. Phlebotomy is also associated with a significant risk of fatal thrombosis. (Burk *et al.*, Semin. Hematol. 23, 132 (1986); Ellis *et al.*, *id.* at 144). Control of erythrocyte production by administration of the c-*kit* antisense oligomers of the present invention is an attractive alternative to phlebotomy and chemotherapy.

The antisense oligonucleotides of the invention are further believed to possess utility in the treatment of hematologic malignancies. Hematologic neoplastic cells believed sensitive to the instant c-

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kit antisense oligonucleotides include, for example, myeloid leukemia cells. The appearance of these cells in the bone marrow and elsewhere in the body is associated with various disease conditions, such as all of the various French-American-British (FAB) subtypes of acute myeloid leukemia, and chronic myeloid leukemia.

The c-kit antisense oligonucleotides are believed particularly useful against acute myelogenous leukemia (AML). Significant activity against chronic myelogenous leukemia (CML) has also been demonstrated. CML, in particular, is characterized by abnormal proliferation of immature granulocytes - neutrophils, eosinophils, and basophils - in the blood, the bone marrow, the spleen, the liver, and sometimes other tissues. The essential feature is accumulation of granulocytic precursors in these tissues. The patient who presents symptoms will characteristically have more than 20,000 white blood cells per μ l, and the count may exceed 400,000. Virtually all CML patients will develop "blast crisis", the terminal stage of the disease during which immature blast cells rapidly proliferate, leading to patient death.

Since c-kit function appears to be important for development of melanocytes, i.e., neural crest-derived pigment cells, and germ (gonadal) cells, it is believed that the antisense oligonucleotides of the present invention are useful for the treatment of malignant melanoma and testicular or ovarian tumors.

The antisense oligonucleotides of the invention find utility as bone marrow purging agents. They may be utilized in vitro to cleanse bone marrow contaminated by hematologic neoplasms. They are believed useful as purging agents in either allogeneic or autologous bone marrow transplantation. They are believed particularly effective in the treatment of hematological malignancies or other neoplasias which metastasize in the bone marrow.

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According to a method for bone marrow purging, bone marrow is harvested from a donor by standard operating room procedures from the iliac bones of the donor. Methods of aspirating bone marrow from donors are well-known in the art. Examples of apparatus and processes for aspirating bone marrow from donors are disclosed in U.S. Patents 4,481,946 and 4,486,188. Sufficient marrow is withdrawn so that the recipient, who is either the donor (autologous transplant) or another individual (allogeneic transplant), may receive from about 4×10^8 to about 8×10^8 processed marrow cells per kg of bodyweight. This generally requires aspiration of about 750 to about 1000 ml of marrow. The aspirated marrow is filtered until a single cell suspension, known to those skilled in the art as a "buffy coat" preparation, is obtained. This suspension of leukocytes is treated with c-kit antisense oligonucleotides in a suitable carrier, advantageously in a concentration of about 8 mg/ml. Alternatively, the leucocyte suspension may be stored in liquid nitrogen using standard procedures known to those skilled in the art until purging is carried out. The purged marrow can be stored frozen in liquid nitrogen until ready for use. Methods of freezing bone marrow and biological substances are disclosed, for example, in U.S. Patents 4,107,937 and 4,117,881.

Other methods of preparing bone marrow for treatment with c-kit antisense may be utilized, which methods may result in even more purified preparations of hematopoietic cells than the aforesaid buffy coat preparation.

One or more hematopoietic growth factors may be added to the aspirated marrow or buffy coat preparation to stimulate growth of hematopoietic neoplasms, and thereby increase their sensitivity to the toxicity of the c-kit antisense oligonucleotides. Such hematopoietic growth factors include, for example, IL-3 and

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granulocyte macrophage colony stimulating factor (GM-CSF). The recombinant human versions of such growth factors are advantageously employed.

After treatment with the antisense oligonucleotides, the cells to be transferred are washed with autologous plasma or buffer to remove unincorporated oligomer. The washed cells are then infused back into the patient.

For in vivo use, the antisense oligonucleotides may be combined with a pharmaceutical carrier, such as a suitable liquid vehicle or excipient and an optional auxiliary additive or additives. The liquid vehicles and excipients are conventional and commercially available. Illustrative thereof are distilled water, physiological saline, aqueous solution of dextrose, and the like. For in vivo antineoplastic use and in vivo erythroid cell reduction, the c-kit mRNA antisense oligonucleotides are preferably administered parenterally, most preferably intravenously. The vehicle is designed accordingly. It is also possible to administer such compounds ex vivo by isolating lymphocytes from peripheral blood, treating them with the antisense oligonucleotides, then returning the treated lymphocytes to the peripheral blood of the donor. Ex vivo techniques have been utilized in treatment of cancer patients with interleukin-2 activated lymphocytes, and are well-known to those skilled in the art.

In addition to administration with conventional carriers, the antisense oligonucleotides may be administered by a variety of specialized oligonucleotide delivery techniques. For example, oligonucleotides may be encapsulated in liposomes for therapeutic delivery. The oligonucleotide, depending upon its solubility, may be present both in the aqueous layer and in the lipidic layer, or in what is generally termed a liposomic suspension. The hydrophobic layer, generally but not exclusively, comprises phospholipids

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such as lecithin and sphingomyelin, steroids such as cholesterol, ionic surfactants such as diacetylphosphate, stearylamine, or phosphatidic acid, and/or other materials of a hydrophobic nature. Oligonucleotides have been successfully encapsulated in unilamellar liposomes.

Reconstituted Sendai virus envelopes have been successfully used to deliver RNA and DNA to cells. Arad et al., Biochem. Biophys. Acta. 859, 88-94 (1986).

The oligonucleotides may be conjugated to poly(L-lysine) to increase cell penetration. Such conjugates are described by Lemaitre et al., Proc. Natl. Acad. Sci. USA, 84, 648-652 (1987). The procedure requires that the 3'-terminal nucleotide be a ribonucleotide. The resulting aldehyde groups are then randomly coupled to the epsilon-amino groups of lysine residues of poly(L-lysine) by Schiff base formation, and then reduced with sodium cyanoborohydride. This procedure converts the 3'-terminal ribose ring into a morpholine structure antisense oligomers.

For ex vivo antineoplastic application, such as, for example, in bone marrow purging, the c-kit antisense oligonucleotides may be administered in amounts effective to kill neoplastic cells while maintaining the viability of normal hematologic cells. Such amounts may vary depending on the nature and extent of the neoplasm, the particular oligonucleotide utilized, the relative sensitivity of the neoplasm to the oligonucleotide, and other factors. Concentrations from about 10 to 200 $\mu\text{g/ml}$ per 10^5 cells may be employed, preferably from about 40 to 150 $\mu\text{g/ml}$ per 10^5 cells. Supplemental dosing of the same or lesser amounts of oligonucleotide are advantageous to optimize the treatment. Thus, for purging bone marrow containing 2×10^7 cell per ml of marrow volume, dosages of from about 2 to 40 mg antisense per ml of marrow may be effectively utilized, preferably from about 8 to 24

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mg/ml. Greater or lesser amounts of oligonucleotide may be employed.

For in vivo use, the c-kit antisense oligonucleotides may be administered in an amount sufficient to result in extracellular concentrations approximating the above stated in vitro concentrations. Preferably, the intracellular concentration is in the range of from about 10 to about 100 μ g/ml. The actual dosage administered may take into account the size and weight of the patient, whether the nature of the treatment is prophylactic or therapeutic in nature, the age, weight, health and sex of the patient, the route of administration, and other factors. Those skilled in the art should be readily able to derive suitable dosages and schedules of administration to suit the specific circumstance. The daily dosage may range from about 0.1 to 1,000 mg oligonucleotide per day, preferably from about 10 to about 1,000 mg per day. Greater or lesser amounts of oligonucleotide may be administered, as required. Those skilled in the art should be readily able to derive appropriate dosages and schedules of administration to suit the specific circumstances and needs of the patient.

The present invention is described in greater detail in the following non-limiting examples.

Example 1

Effect of c-kit Antisense Oligomer Exposure on Normal Hematopoietic Progenitor Cell Growth.

The effect of c-kit antisense oligonucleotide on hematopoietic progenitor cell cloning efficiency and development was systematically investigated by assessing CFU-E, burst-forming unit-erythroid (BFU-E), CFU-GM, and CFU-MEG growth after oligomer exposure.

Cells: Human bone marrow cells (BMC) were obtained from normal, healthy volunteers by Ficoll-

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Hypaque density gradient centrifugation, and were partially enriched for hematopoietic progenitors by removal of adherent, phagocytic elements and T lymphocytes (Gewirtz *et al.*, *J. Immunol.* 139, 2915-2925 (1987)). For some experiments, the adherent, T lymphocyte-depleted population (A⁺TMNC) was further enriched by positively selecting CD34⁺ cells with immunomagnetic beads (Dynal A.S., Oslo, Norway). The A⁺TMNC cells were suspended in supplemented alpha medium and incubated with mouse anti-HPCA-I antibody in 1:20 dilution, 45 minutes, at 4°C with gentle inverting of tubes. Cells were washed x 3 in Supplemented alpha medium, and then incubated with beads coated with the Fc fragment of goat anti-mouse IgG₁ (75 µl of immunobeads/10⁷ A⁺TMNC). After 45 minutes of incubation (4°C), cells adherent to the beads were positively selected using a magnetic particle concentrator as directed by the manufacturer.

Oligodeoxynucleotides: Unmodified, 18-nucleotide oligodeoxynucleotides (oligomers) were synthesized as previously reported (Gewirtz *et al.*, *Science* 242, 1303-1306 (1988)). In brief, oligomers were synthesized on an Applied Biosystems 380B DNA synthesizer by means of a β-cyanoethyl phosphoramidite chemistry. Oligomers were purified by ethanol precipitation and multiple washes in 70% ethanol. They were then lyophilized to dryness and redissolved in culture medium prior to use at a concentration of 1µg/µl (0.175 µM). Oligomer sequences employed, corresponding to codons 1-6 of the published human c-kit cDNA sequence (Yarden, *et al.*, *The EMBO Journal* 6, 3341-3351 (1987)), were as follows: ATGAGAGGCG CTCGCGGC (SEQ ID NO:14), sense oligomer; GCCGCGAGCG CCTCTCAT (SEQ ID NO:10), antisense oligomer; and GCACCGTCTG CCAGTCGC (SEQ ID NO:15), scrambled sequence oligomer.

Oligomer Treatment of Cells: Cells were exposed to oligomers as previously described (Gewirtz

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et al., Science 242, 1303-1306 (1988)). 2×10^5 A⁺T⁺MNC or CD34⁺MNC were incubated in 5 ml polypropylene tubes (Fisher Scientific, Pittsburgh, PA) in a total volume of 0.4 ml of Iscove's modified Dulbecco's medium (IMDM) containing 2% human AB serum and 10 mM Hepes buffer. Oligomers were added at time zero (2.5-100 μ g/ml), and 50% of the initial dose was added again 18 hours later (final total concentration ~0.6-26 μ M). Twenty-four hours after the first addition of oligomers, cells were prepared for plating in plasma clot or methylcellulose cultures. Cells (1×10^5 A⁺T⁺MNC or 1×10^4 CD34⁺MNC per dish) were not washed before plating. Control cultures were manipulated in an identical manner but were not treated with oligomers.

Colony Assays: Assays for hematopoietic progenitor cells of varying lineages were carried out essentially as reported (Id.). In brief, cells (10^5 A⁺T⁺MNC or 5×10^3 CD34⁺MNC) were resuspended in IMDM supplemented with 30% human AB serum, 1% BSA, 10^{-4} M mercaptoethanol, and 10% citrated bovine plasma (Hyclone Laboratories, Denver, CO). Addition of the appropriate recombinant human growth factors allowed for stimulation of the following cell types:

CFU-E: 5 U/ml EPO;

BFU-E: 20 U/ml IL-3 and 5 U/ml EPO, or
100 ng/ml SCF and 5 U/ml EPO;

CFU-GM: 20 U/ml IL-3 and 5 ng/ml granulocyte-macrophage colony stimulating factor;

CFU-MEG: 20 U/ml IL-3 and 100 ng/ml IL-6.

One ml volumes were cultured in 35 mm petri dishes at 37°C, 5% CO₂, and 95% humidity. CFU-E colonies were scored at day 7, BFU-E colonies at day 14, CFU-MEG at day 12, and CFU-GM at day 11 of incubation. Colony identification was carried out as previously described (Id.).

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5 Statistics: Statistical significance of differences between means of test groups was assessed by Mann-Whitney non-parametric analysis using a statistical software package (Statview 512+, BrainPower, Inc., Calabasas, CA). The results appear in Tables 1 and 2. Values presented are actual colonies counted, pooled from two or three individual studies.

Table 1

**Effect of c-kit on A^T cell
derived colony formation**

Progenitor Cell Type	Control	Sense	Scrambled Sequence	Antisense
CFU-E	182, 209	153, 142	119, 128	33, 59
	1943, 543	1635, 1135	627, 649	243, 213
	148, 100	129, 176	149, 206	97, 107
	Mean (\pm SE)=	Mean (\pm SE)=	Mean (\pm SE)=	Mean (\pm SE)=
	522 \pm 291	562 \pm 268	313 \pm 103	125 \pm 34
BFU-E	133, 152	117, 106	94, 64	60, 149
	534, 392	601, 249	273, 246	126, 113
	206, 172	215, 258	162, 246	59, 51
	Mean (\pm SE)=	Mean (\pm SE)=	Mean (\pm SE)=	Mean (\pm SE)=
	265 \pm 66	258 \pm 74	181 \pm 36	76 \pm 14
CFU-GM	212, 189	231, 179	282, 193	195, 220
	412, 408	395, 421	457, 384	407, 471
	217, 241	230, 237	201, 199	293, 187
	209, 246	Mean (\pm SE)=	Mean (\pm SE)=	Mean (\pm SE)=
	Mean (\pm SE)=	282 \pm 41	286 \pm 46	296 \pm 49
	280 \pm 42			
CFU-MEG	114, 107	133, 117	154, 113	127, 112
	93, 100	58, 52	53, 40	47, 54
	Mean (\pm SE)=	Mean (\pm SE)=	Mean (\pm SE)=	Mean (\pm SE)=
	104 \pm 5	90 \pm 20	90 \pm 27	85 \pm 20

Table 2

Effect of c-kit oligomers on CD34⁺
dell derived colony formation

Progenitor Cell Type	Control	Sense	Scrambled Sequence	Antisense
CFU-E	5, 9	16, 14	6, 21	1, 0
	16, 14	11, 17	22, 8	0, 0
	71, 79	78, 48	86, 91	40, 22
	Mean (\pm SE)=	Mean (\pm SE)=	Mean (\pm SE)=	Mean (\pm SE)=
	32 \pm 12	31 \pm 11	39 \pm 15	11 \pm 7
BFU-E	179, 229	293, 120	191, 261	81, 75
	276, 281	241, 151	227, 283	34, 91
	271, 451	440, 361	321, 351	92, 111
	Mean (\pm SE)=	Mean (\pm SE)=	Mean (\pm SE)=	Mean (\pm SE)=
	281 \pm 37	286 \pm 50	272 \pm 24	81 \pm 11
CFU-GM	321, 299	287, 360	321, 339	354, 319
	309, 312	316, 262	289, 324	349, 271
	114, 121	109, 103	84, 106	94, 103
	90, 135	Mean (\pm SE)=	Mean (\pm SE)=	Mean (\pm SE)=
	Mean (\pm SE)=	240 \pm 44	244 \pm 48	248 \pm 49
	213 \pm 37			

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As shown in Table 1, when A^TMNC were employed as indicator cells, c-kit antisense oligomers inhibited growth of CFU-E ~75%, and BFU-E ~71%, when employed at the highest doses. Inhibition was sequence-specific since neither sense, nor scrambled sequence oligomers, significantly affected colony growth in comparison to untreated controls. In contrast to these results, CFU-GM and CFU-MEG derived colony formation was unaffected by exposure to any of the oligomers, at any of the doses employed (Table 1).

Similar results were obtained after exposure of CD34⁺ cells to c-kit oligomers. As shown in Table 2, the mean number of CFU-E colonies decreased by ~66% after exposure to c-kit antisense oligomers while the number of BFU-E colonies decreased ~71%. Neither sense nor scrambled sequence oligomers inhibited colony formation. As was also noted with the less purified cell preparation, neither antisense nor control oligomers inhibited CFU-GM colony formation. The failure of c-kit antisense to inhibit CFU-GM and CFU-MEG is surprising since W/W^v mice have been reported to have defective granulopoiesis and megakaryocytopoiesis (Chervenick *et al.*, Proc. Soc. Exp. Biol. Med. 152, 398-402 (1976)).

Erythroid colony formation was inhibited in a dose-dependent fashion. See Fig. 3, showing the effect of various concentrations of c-kit antisense oligomer on BFU-E-derived colony formation. Moreover, residual colonies were much smaller for the antisense-treated cells versus untreated controls (data not shown).

Reverse Transcription-Polymerase Chain Reaction (RT-PCR): As additional proof that the antisense effect was due to a specific decrement in c-kit mRNA levels, the kinetics of c-kit message expression in marrow mononuclear cells were examined, and the effect of oligomer exposure on c-kit mRNA levels was then assessed by the following RT-PCR procedure.

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Total RNA was extracted from cells using the guanidine isothiocyanate method of Chirgwin et al., Biochemistry, 18, 5294 (1979). Cells ($2-5 \times 10^6$) were lysed in 250 μ l of guanidine thiocyanate buffer (4M guanidine thiocyanate; 50 mM sodium acetate pH 5.0; 1 mM EDTA; 1M β -mercaptoethanol; 0.5% sarcosyl) and then layered over 250 μ l of a cesium chloride (5.7 M) cushion in Beckman open-top ultra clear centrifuge tubes (0.8 ml). Tubes were centrifuged (Beckman TL-100 Ultracentrifuge; 100,000 RMP; 1.5 hours; 20°C) and the resulting RNA pellet was resuspended in ~400 μ l of water, precipitated with 0.3 M potassium acetate, washed twice in 75% ethanol, and then stored at -70°C until used.

RNA was reverse-transcribed with 500 U of Moloney murine leukemia virus reverse transcriptase (MoMLV-RT) and 50 pmol of a 22-nucleotide oligodeoxynucleotide 3' primer complementary to nucleotides 1201-1179 (CTAGG-AATGT GTAAGTGCCT CC, SEQ ID NO:16) of the published c-kit cDNA sequence. The resulting cDNA fragment was amplified using 5 U of Thermus aquaticus (Tag) polymerase and a 22-nucleotide oligodeoxynucleotide 5' primer specific for c-kit nucleotides 842-864 (GGTTGACTAT CAGTTCA-GCG AG, SEQ ID NO:17). Twenty-five μ l of amplified product was electrophoresed on 4% agarose gel and subsequently transferred to a nylon filter. Filters were pre-hybridized, and then probed with a 32 P end-labeled oligonucleotide probe (Caracciolo et al., Science 245, 1107-1109 (1989)) corresponding to the 21-nucleotide c-kit sequence (GATCCACTGC TGGTGTTCAG G, SEQ ID NO:18) contained within the amplified region (nucleotides 1068-1047). Autoradiography was performed by exposing filters on Kodak X-ray film at -70°C using intensifying screens.

A⁺TMNC cells were kept for 24 hours at 4°C in IMDM containing 2% human AB serum, then shifted to 37°C and stimulated with IL-3 (20 U/ml) and EPO (5 U/ml) in 5% human AB serum. C-kit expression was determined at

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intervals according to RT-PCR. The results are indicated in Figure 1. The Figure 1 lanes indicate relative c-kit transcript amounts determined by autoradiography of RT-PCR hybridization gels at the following intervals after stimulation:

	<u>Fig. 1 Lane</u>	<u>Time to RT-PCR Assay (hrs)</u>
	1	0
10	2	2
	3	8
	4	12
	5	24
	6	36
15	7	48
	9	H ₂ O

Lane 8 contained H₂O as a control. Expression appeared to peak at ~36 hours.

A^TMNC cells stimulated (t=0) in the same manner were exposed to c-kit sense, antisense or mismatch oligomers. c-kit expression was assayed by RT-PCR (t=36 hrs) as above. The results appear in Figure 2. The lanes are identified as follows:

	<u>Fig.2 Lane</u>	<u>Treatment</u>
	1	control cells (t=0)
	2	control cells (t=36 hrs)
	3	sense (t=36 hrs)
30	4	antisense (t=36 hrs)
	5	mismatch (t=36 hrs)

Antisense-treated cells (lane 4) had no detectable c-kit mRNA, while sense (lane 3) and scrambled sequence (lane 5) treated cells had levels which were similar to those observed in untreated control cells at the same time point (lane 2).

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Example 2Effect of c-kit Antisense Oligomer Exposure on
Malignant Hematopoietic Progenitor Cell Growth

5 To explore the importance of the c-kit receptor
in regulating malignant hematopoietic cell growth, we
employed a strategy which we have successfully employed
in the past (Calabretta et al., Proc. Nat. Acad. Sci.
10 USA, 88, 2351 (1991)), and which is essentially identical
to that described above. A⁺T⁺MNC were obtained from
patients with a variety of hematologic malignancies and
exposed to the c-kit oligomers utilized in the preceding
normal cell studies. Effects on the ability of malignant
15 CFU-GM to form colonies, an index of the effect on
malignant cell survival and proliferative activity, was
then assessed. A total of twenty-two patients were
studied, three with acute lymphocytic leukemia, four
with acute myelogenous leukemia, ten with chronic myelo-
20 genous leukemia, and one with a myelodysplastic (pre-
leukemic) syndrome and four with polycythemia vera.
Overall response rates, and response rate by disease
type, is given in Table 3.

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Table 3

**Effect of c-kit oligomers on growth of malignant
hematopoietic colony forming cells**

5		Disease Type	No. Pts	Responders	% Decrease Colonies	Non- Respon- ders
10		Acute lymphocytic leukemia	3	1	68%	2
15		Acute myelogenous leukemia	4	1	63%	3
20		Chronic myelogenous leukemia	10	5	84% 33% 40% 90% 78% Mean=65%±26%	5
25		Myelodysplastic syndrome	1	1	68%	-
30		Polycythemia vera	4	4	Mean 74%±24%	-
		TOTAL	22	12	66%	10

While the number of patients in categories other than CML is small, the data nonetheless suggest that patients with CML are particularly likely to respond to c-kit antisense. Accordingly, c-kit antisense oligomers are believed particularly useful as CML bone marrow purging agents. In addition, because of their marked inhibition of erythroid progenitor cells, it is believed that c-kit oligomers are useful in controlling the markedly elevated hemoglobin/hematocrit found in patients with PV, another myeloproliferative disorder.

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Example 3Effect of c-kit Antisense Oligomer Exposure
on BFU-E Responsiveness to Stem Cell Factor

5 To provide proof that c-kit antisense mediated inhibition of erythropoiesis was due to the absence of KIT receptor function, we sought to demonstrate that BFU-E responsiveness to stem cell factor (SCF) could be abolished in a sequence-specified manner after exposure
10 to c-kit oligomers. Accordingly, CD34⁺ MNC (2×10^4) were cloned in the presence of 5 units of EPO and 100 ng of SCF per ml alone or with sense, antisense, or scrambled-sequence c-kit oligomers (final concentration, 150 μ g/ml ($\sim 26 \mu$ M)). In four experiments, 191 ± 19 BFU-E
15 (mean \pm SD) were grown in the presence of the growth factors alone. These numbers were not statistically different from those cloned with sense (183 ± 29 ; $P = 0.654$) or scrambled-sequence oligomers (180 ± 20 ; $P = 0.758$). In the presence of the c-kit antisense oligo-
20 mers, BFU-E-derived colony formation was completely abolished (0.4 ± 0.7 ; $P < 0.0001$), suggesting that KIT receptor was no longer present to interact with its ligand.

25 The following non-limiting example illustrates one methodology for bone marrow purging according to the present invention.

Example 4Bone Marrow Purging with c-kit
30 Antisense Oligonucleotide

35 Bone marrow is harvested from the iliac bones of a donor under general anesthesia in an operating room using standard techniques. Multiple aspirations are taken into heparinized syringes. Sufficient marrow is withdrawn so that the marrow recipient will be able to receive about 4×10^8 to about 8×10^8 processed marrow

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cells per kg of body weight. Thus, about 750 to 1000 ml of marrow is withdrawn. The aspirated marrow is transferred immediately into a transport medium (TC-199, Gibco, Grand Island, New York) containing 10,000 units of preservative-free heparin per 100 ml of medium. The aspirated marrow is filtered through three progressively finer meshes until a single cell suspension results, i.e., a suspension devoid of cellular aggregates, debris and bone particles. The filtered marrow is then processed further into an automated cell separator (e.g., Cobe 2991 Cell Processor) which prepares a "buffy coat" product, (i.e., leukocytes devoid of red cells and platelets). The buffy coat preparation is then placed in a transfer pack for further processing and storage. It may be stored until purging in liquid nitrogen using standard procedures. Alternatively, purging can be carried out immediately, then the purged marrow may be stored frozen in liquid nitrogen until it is ready for transplantation.

The purging procedure may be carried out as follows. Cells in the buffy coat preparation are adjusted to a cell concentration of about $2 \times 10^7/\text{ml}$ in TC-199 containing about 20% autologous plasma. C-kit antisense oligodeoxynucleotide, for example, in a concentration of about 8 mg/ml, is added to the transfer packs containing the cell suspension. Recombinant human hematopoietic growth factors, e.g., rH IL-3 or rH GM-CSF, may be added to the suspension to stimulate growth of hematopoietic neoplasms and thereby increase their sensitivity c-kit antisense oligonucleotide toxicity. The transfer packs are then placed in a 37°C waterbath and incubated for 18 - 24 hours with gentle shaking. The cells may then either be frozen in liquid nitrogen or washed once at 4°C in TC-199 containing about 20% autologous plasma to remove unincorporated oligomer. Washed cells are then infused into the recipient. Care must be taken to work under sterile conditions wherever possible

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and to maintain scrupulous aseptic techniques at all times.

5 The present invention may be embodied in other specific forms without departing from the spirit or essential attributes thereof and, accordingly, reference should be made to the appended claims, rather than to the foregoing specification, as indicating the scope of the invention.

10 All references cited herein with respect to synthetic, preparative and analytical procedures are incorporated herein by reference.

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SEQUENCE LISTING**(1) GENERAL INFORMATION:****(i) APPLICANT:** Gewirtz, Alan M.

5

Calabretta, Bruno

(ii) TITLE OF INVENTION: Antisense Oligonucleotides to c-kit Proto-Oncogene and Uses Thereof**(iii) NUMBER OF SEQUENCES:** 18**(iv) CORRESPONDENCE ADDRESS:**

10

(A) ADDRESSEE: Temple University - Of The Commonwealth System of Higher Education**(B) STREET:** 406 University Services Building**(C) CITY:** Philadelphia**(D) STATE:** Pennsylvania

15

(E) COUNTRY: U.S.A.**(F) ZIP:** 19122**(v) COMPUTER READABLE FORM:****(A) MEDIUM TYPE:** Diskette, 3.50 inch, 720 Kb**(B) COMPUTER:** IBM PS/2

20

(C) OPERATING SYSTEM: MS-DOS**(D) SOFTWARE:** WordPerfect 5.1**(vi) CURRENT APPLICATION DATA:****(A) APPLICATION NUMBER:****(B) FILING DATE:**

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30

(A) NAME: Monaco, Daniel A.**(B) REGISTRATION NUMBER:** 30,480**(C) REFERENCE/DOCKET NUMBER:** 6056-129 PC**(ix) TELECOMMUNICATION INFORMATION:****(A) TELEPHONE:** (215) 568-8383

35

(B) TELEFAX: (215) 568-5549**(C) TELEX:** None

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(2) INFORMATION FOR SEQ ID NO:1:

(i) SEQUENCE CHARACTERISTICS:

- (A) LENGTH: 40 Nucleotides
(B) TYPE: nucleic acid
(C) STRANDEDNESS: single stranded
(D) TOPOLOGY: linear

(xi) SEQUENCE DESCRIPTION: SEQ ID NO:1:

GAACGCAGAG AAAATCCCAG GCGCCGCGAG CGCCTCTCAT 40

(2) INFORMATION FOR SEQ ID NO:2:

(i) SEQUENCE CHARACTERISTICS:

- (A) LENGTH: 26 Nucleotides
(B) TYPE: nucleic acid
(C) STRANDEDNESS: single stranded
(D) TOPOLOGY: linear

(xi) SEQUENCE DESCRIPTION: SEQ ID NO:2:

TCCCAGGCGC CGCGAGCGCC TCTCAT 26

(2) INFORMATION FOR SEQ ID NO:3:

(i) SEQUENCE CHARACTERISTICS:

- (A) LENGTH: 25 Nucleotides
(B) TYPE: nucleic acid
(C) STRANDEDNESS: single stranded
(D) TOPOLOGY: linear

(xi) SEQUENCE DESCRIPTION: SEQ ID NO:3:

CCCAGGCGCC GCGAGCGCCT CTCAT 25

(2) INFORMATION FOR SEQ ID NO:4:

(i) SEQUENCE CHARACTERISTICS:

- (A) LENGTH: 24 Nucleotides
(B) TYPE: nucleic acid
(C) STRANDEDNESS: single stranded
(D) TOPOLOGY: linear

(xi) SEQUENCE DESCRIPTION: SEQ ID NO:4:

CCAGGCGCCG CGAGCGCCTC TCAT 24

(2) INFORMATION FOR SEQ ID NO:5:

- 35 -

(i) **SEQUENCE CHARACTERISTICS:**

- (A) **LENGTH:** 23 Nucleotides
(B) **TYPE:** nucleic acid
(C) **STRANDEDNESS:** single stranded
(D) **TOPOLOGY:** linear

(xi) **SEQUENCE DESCRIPTION: SEQ ID NO:5:**
CAGGCGCCGC GAGCGCCTCT CAT 23

(2) **INFORMATION FOR SEQ ID NO:6:**(i) **SEQUENCE CHARACTERISTICS:**

- (A) **LENGTH:** 22 Nucleotides
(B) **TYPE:** nucleic acid
(C) **STRANDEDNESS:** single stranded
(D) **TOPOLOGY:** linear

(xi) **SEQUENCE DESCRIPTION: SEQ ID NO:6:**
AGGCGCCGCG AGCGCCTCTC AT 22

(2) **INFORMATION FOR SEQ ID NO:7:**(i) **SEQUENCE CHARACTERISTICS:**

- (A) **LENGTH:** 21 Nucleotides
(B) **TYPE:** nucleic acid
(C) **STRANDEDNESS:** single stranded
(D) **TOPOLOGY:** linear

(xi) **SEQUENCE DESCRIPTION: SEQ ID NO:7:**
GGCGCCGCGA GCGCCTCTCA T 21

(2) **INFORMATION FOR SEQ ID NO:8:**(i) **SEQUENCE CHARACTERISTICS:**

- (A) **LENGTH:** 20 Nucleotides
(B) **TYPE:** nucleic acid
(C) **STRANDEDNESS:** single stranded
(D) **TOPOLOGY:** linear

(xi) **SEQUENCE DESCRIPTION: SEQ ID NO:8:**
GCGCCGCGAG CGCCTCTCAT 20

(2) **INFORMATION FOR SEQ ID NO:9:**(i) **SEQUENCE CHARACTERISTICS:**

- 36 -

- (A) **LENGTH:** 19 Nucleotides
- (B) **TYPE:** nucleic acid
- (C) **STRANDEDNESS:** single stranded
- (D) **TOPOLOGY:** linear

5 (xi) **SEQUENCE DESCRIPTION: SEQ ID NO:9:**
CGCCGCGAGC GCCTCTCAT 19

(2) **INFORMATION FOR SEQ ID NO:10:**

(i) **SEQUENCE CHARACTERISTICS:**

- 10 (A) **LENGTH:** 18 Nucleotides
- (B) **TYPE:** nucleic acid
- (C) **STRANDEDNESS:** single stranded
- (D) **TOPOLOGY:** linear

15 (xi) **SEQUENCE DESCRIPTION: SEQ ID NO:10:**
GCCGCGAGCG CCTCTCAT 18

(2) **INFORMATION FOR SEQ ID NO:11:**

(i) **SEQUENCE CHARACTERISTICS:**

- 20 (A) **LENGTH:** 17 Nucleotides
- (B) **TYPE:** nucleic acid
- (C) **STRANDEDNESS:** single stranded
- (D) **TOPOLOGY:** linear

(xi) **SEQUENCE DESCRIPTION: SEQ ID NO:11:**
CCGCGAGCGC CTCTCAT 17

25

(2) **INFORMATION FOR SEQ ID NO:12:**

(i) **SEQUENCE CHARACTERISTICS:**

- 30 (A) **LENGTH:** 16 Nucleotides
- (B) **TYPE:** nucleic acid
- (C) **STRANDEDNESS:** single stranded
- (D) **TOPOLOGY:** linear

(xi) **SEQUENCE DESCRIPTION: SEQ ID NO:12:**
CGCGAGCGCC TCTCAT 16

35

(2) **INFORMATION FOR SEQ ID NO:13:**

(i) **SEQUENCE CHARACTERISTICS:**

- (A) **LENGTH:** 15 Nucleotides

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- (B) TYPE: nucleic acid
- (C) STRANDEDNESS: single stranded
- (D) TOPOLOGY: linear

(xi) SEQUENCE DESCRIPTION: SEQ ID NO:13:
GCGAGCGCCT CTCAT 15

(2) INFORMATION FOR SEQ ID NO:14:

(i) SEQUENCE CHARACTERISTICS:

- (A) LENGTH: 18 Nucleotides
- (B) TYPE: nucleic acid
- (C) STRANDEDNESS: single stranded
- (D) TOPOLOGY: linear

(xi) SEQUENCE DESCRIPTION: SEQ ID NO:14:
ATGAGAGGCG CTCGCGGC 18

(2) INFORMATION FOR SEQ ID NO:15:

(i) SEQUENCE CHARACTERISTICS:

- (A) LENGTH: 18 Nucleotides
- (B) TYPE: nucleic acid
- (C) STRANDEDNESS: single stranded
- (D) TOPOLOGY: linear

(xi) SEQUENCE DESCRIPTION: SEQ ID NO:15:
GCACCGTCTG CCAGTCGC 18

(2) INFORMATION FOR SEQ ID NO:16:

(i) SEQUENCE CHARACTERISTICS:

- (A) LENGTH: 22 Nucleotides
- (B) TYPE: nucleic acid
- (C) STRANDEDNESS: single stranded
- (D) TOPOLOGY: linear

(xi) SEQUENCE DESCRIPTION: SEQ ID NO:16:
CTAGGAATGT GTAAGTGCCT CC 22

(2) INFORMATION FOR SEQ ID NO:17:

(i) SEQUENCE CHARACTERISTICS:

- (A) LENGTH: 22 Nucleotides
- (B) TYPE: nucleic acid

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(C) STRANDEDNESS: single stranded

(D) TOPOLOGY: linear

(xi) SEQUENCE DESCRIPTION: SEQ ID NO:17:
GGTTGACTAT CAGTTCAGCG AG 22

5

(2) INFORMATION FOR SEQ ID NO:18:

(i) SEQUENCE CHARACTERISTICS:

(A) LENGTH: 21 Nucleotides

(B) TYPE: nucleic acid

(C) STRANDEDNESS: single stranded

(D) TOPOLOGY: linear

(xi) SEQUENCE DESCRIPTION: SEQ ID NO:18:
GATCCACTGC TGGTGTTCAG G 21

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CLAIMS

1. A pharmaceutical composition comprising a pharmaceutical carrier and an oligonucleotide which has a nucleotide sequence complementary to at least a portion of the mRNA transcript of the human c-kit gene, said oligonucleotide being hybridizable to said mRNA transcript.

2. A composition according to claim 1 wherein the oligonucleotide comprises an at least 12-mer.

3. A composition according to claim 2 wherein the oligonucleotide has a nucleotide sequence complementary to a portion of the c-kit mRNA lying within about 40 nucleotides of the translation initiation codon.

4. A composition according to claim 2 wherein the oligonucleotide is an oligodeoxynucleotide having a deoxynucleotide sequence complementary to a portion of the c-kit mRNA transcript including the translation initiation codon of said transcript.

5. A composition according to claim 2 wherein the oligonucleotide comprises from a 12-mer to a 40-mer oligodeoxynucleotide.

6. A composition according to claim 5 wherein the oligodeoxynucleotide is an alkylphosphonate oligodeoxynucleotide or a phosphorotioate oligodeoxynucleotide.

7. A composition according to claim 5 wherein the oligodeoxynucleotide comprises from a 15-mer to a 30-mer.

8. A composition according to claim 7 wherein the oligodeoxynucleotide comprises from an 18-mer to a 26-mer.

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9. A composition according to claim 8 wherein the oligodeoxynucleotide comprises from an 18-mer to a 21-mer.

10. A composition according to claim 7 wherein the oligodeoxynucleotide has a sequence selected from the group of sequences consisting of:

SEQ ID NO:2,
SEQ ID NO:3,
SEQ ID NO:4,
SEQ ID NO:5,
SEQ ID NO:6,
SEQ ID NO:7,
SEQ ID NO:8,
SEQ ID NO:9,
SEQ ID NO:10,
SEQ ID NO:11,
SEQ ID NO:12, and
SEQ ID NO:13.

11. A composition according to claim 10 wherein the oligodeoxynucleotide has a nucleotide sequence of SEQ ID NO:10.

12. An oligonucleotide which has a nucleotide sequence complementary to at least a portion of the mRNA transcript of the human c-kit gene, said oligonucleotide being hybridizable to said mRNA transcript.

13. An oligonucleotide according to claim 12 which comprises an at least 12-mer.

14. An oligonucleotide according to claim 13 having a nucleotide sequence complementary to a portion of the c-kit mRNA lying within about 40 nucleotides of the translation initiation codon.

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15. An oligodeoxynucleotide according to claim 13 which is an oligodeoxynucleotide having a deoxynucleotide sequence complementary to a portion of the c-kit mRNA transcript including the translation initiation codon of said transcript.

16. An oligodeoxynucleotide according to claim 13 wherein the oligonucleotide comprises from a 12-mer to a 40-mer oligodeoxynucleotide.

17. An oligodeoxynucleotide according to claim 16 which is an alkylphosphonate oligodeoxynucleoside or a phosphorothioate oligodeoxynucleotide.

18. An oligodeoxynucleotide according to claim 16 which comprises from a 15-mer to a 30-mer.

19. An oligodeoxynucleotide according to claim 18 which comprises from a 18-mer to a 26-mer.

20. An oligodeoxynucleotide according to claim 19 which comprises from a 18-mer to a 21-mer.

21. An oligodeoxynucleotide according to claim 16 selected from the group of oligodeoxynucleotides having sequences consisting of:

SEQ ID NO:2,
SEQ ID NO:3,
SEQ ID NO:4,
SEQ ID NO:5,
SEQ ID NO:6,
SEQ ID NO:7,
SEQ ID NO:8,
SEQ ID NO:9,
SEQ ID NO:10,
SEQ ID NO:11,
SEQ ID NO:12, and

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SEQ ID NO: 13.

22. An oligodeoxynucleotide according to claim 21 having the nucleotide sequence SEQ ID NO:10.

23. A method for in vivo or ex vivo treatment of hematologic neoplasms characterized by c-kit expression comprising administering to a host in need of such treatment, or to cells harvested from such host, an effective amount of an oligonucleotide which has a nucleotide sequence complementary to at least a portion of the mRNA transcript of the human c-kit gene, said oligonucleotide being hybridizable to said mRNA transcript.

24. The method according to claim 23 wherein the oligonucleotide is an at least 12-mer.

25. A method according to claim 24 wherein the oligonucleotide has a nucleotide sequence complementary to a portion of the c-kit mRNA lying within about 40 nucleotides of the translation initiation codon.

26. A method according to claim 24 wherein the oligodeoxynucleotide is an oligodeoxynucleotide having a deoxynucleotide sequence complementary to a portion of the c-kit mRNA transcript including the translation initiation codon of said transcript.

27. A method according to claim 24 wherein the oligonucleotide comprises from a 12-mer to a 40-mer oligodeoxynucleotide.

28. A method according to claim 27 wherein the oligodeoxynucleotide is an alkylphosphonate oligodeoxynucleoside or a phosphorothioate oligodeoxynucleotide.

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29. A method according to claim 27 wherein the oligodeoxynucleotide is selected from the group of oligodeoxynucleotides having sequences consisting of:

SEQ ID NO:2,
SEQ ID NO:3,
SEQ ID NO:4,
SEQ ID NO:5,
SEQ ID NO:6,
SEQ ID NO:7,
SEQ ID NO:8,
SEQ ID NO:9,
SEQ ID NO:10,
SEQ ID NO:11,
SEQ ID NO:12, and
SEQ ID NO: 13.

30. A method according to any of claims 23, 24, 25, 26, 27, 28 or 29 comprising treating aspirated bone marrow cells and returning the aspirated cells to the host following treatment.

31. A method according to claim 23 wherein the hematologic neoplasm comprises chronic myelogenous leukemia.

32. A method according to claim 23 wherein the hematologic neoplasm comprises acute myelogenous leukemia.

33. A method for inhibiting proliferation of erythroid cells comprising administering to a host an effective amount of an oligonucleotide which has a nucleotide sequence complementary to at least a portion of the mRNA transcript of the human c-kit gene, said oligonucleotide being hybridizable to said mRNA transcript.

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34. A method according to claim 33 wherein the oligonucleotide is an at least 12-mer.

35. A method according to claim 34 wherein the oligonucleotide has a nucleotide sequence complementary to a portion of the c-kit mRNA lying within about 40 nucleotides of the translation initiation codon.

36. A method according to claim 34 wherein the oligonucleotide is an oligodeoxynucleotide having a deoxynucleotide sequence complementary to a portion of the c-kit mRNA transcript including the translation initiation codon of said transcript.

37. A method according to claim 34 wherein the oligonucleotide comprises from a 12-mer to a 40-mer oligodeoxynucleotide.

38. A method according to claim 37 wherein the oligodeoxynucleotide is an alkylphosphonate oligodeoxynucleoside or a phosphorothioate oligodeoxynucleotide.

39. A method according to claim 37 wherein the oligodeoxynucleotide is from a 15-mer to a 30-mer.

40. A method according to claim 39 wherein the oligodeoxynucleotide is from an 18-mer to a 26-mer.

41. A method according to claim 40 wherein the oligodeoxynucleotide is from an 18-mer to a 21-mer.

42. A method according to claim 41 wherein the oligodeoxynucleotide has a nucleotide sequence selected from the group of sequences consisting of:

SEQ ID NO:2,

SEQ ID NO:3,

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SEQ ID NO:4,
SEQ ID NO:5,
SEQ ID NO:6,
SEQ ID NO:7,
SEQ ID NO:8,
SEQ ID NO:9,
SEQ ID NO:10,
SEQ ID NO:11,
SEQ ID NO:12, and
SEQ ID NO: 13.

43. A method for treating malignant melanoma comprising administering to a host in need thereof an effective amount of an oligonucleotide which has a nucleotide sequence complementary to at least a portion of the mRNA transcript of the human c-kit gene, said oligonucleotide being hybridizable to said mRNA transcript.

44. A method according to claim 43 wherein the oligonucleotide comprises an at least 12-mer.

45. A method according to claim 44 wherein the oligonucleotide comprises from a 12-mer to a 40-mer oligodeoxynucleotide.

46. A method according to claim 45 wherein the oligonucleotide has a nucleotide sequence complementary to a portion of the c-kit mRNA lying within about 40 nucleotides of the translation initiation codon.

47. A method according to claim 45 wherein the oligonucleotide is an alkylphosphonate oligodeoxynucleoside or a phosphorothioate oligodeoxynucleotide.

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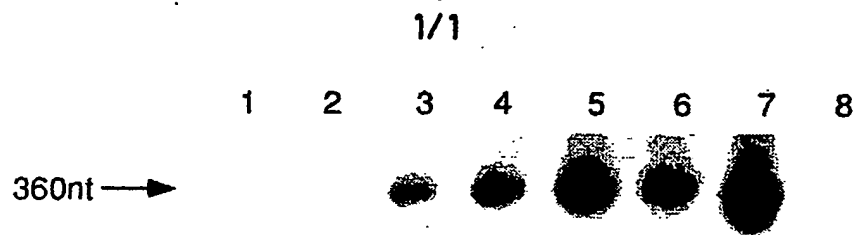
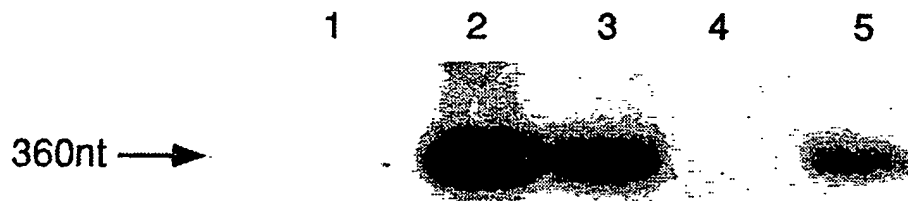
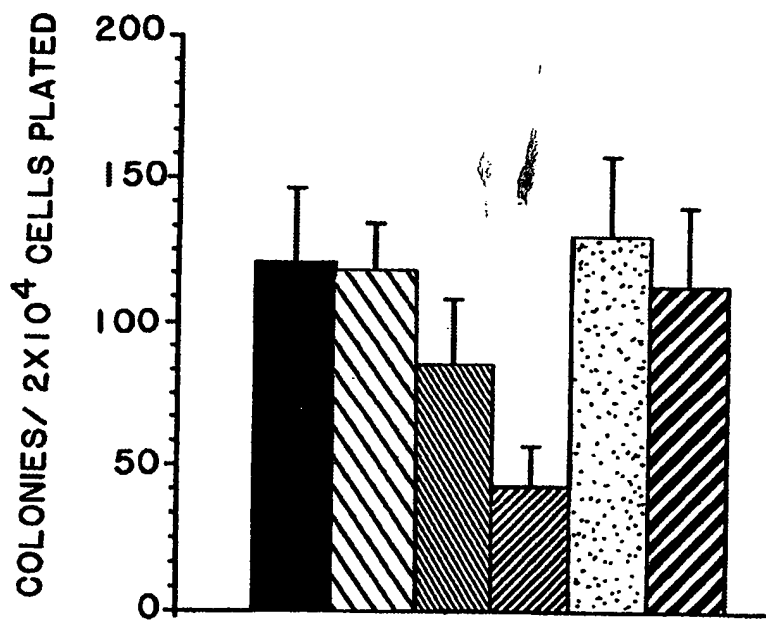
48. A method for treating testicular or ovarian tumors comprising administering to a host in need thereof an effective amount of an oligonucleotide which has a nucleotide sequence complementary to at least a portion of the mRNA transcript of the human c-kit gene, said oligonucleotide being hybridizable to said mRNA transcript.

49. A method according to claim 48 wherein the oligonucleotide comprises an at least 12-mer.

50. A method according to claim 49 wherein the oligonucleotide comprises from a 12-mer to a 40-mer oligodeoxynucleotide.

51. A method according to claim 50 wherein the oligonucleotide has a nucleotide sequence complementary to a portion of the c-kit mRNA lying within about 40 nucleotides of the translation initiation codon.

52. A method according to claim 50 wherein the oligonucleotide is an alkylphosphonate oligodeoxynucleoside or a phosphorothioate oligodeoxynucleotide.

*FIG. 1**FIG. 2**FIG. 3*

INTERNATIONAL SEARCH REPORT

International application No.

PCT/US92/02854

A. CLASSIFICATION OF SUBJECT MATTER

IPC(5) :A61K 37/00, 31/70; C07H 15/12

US CL :424/93U; 514/44, 908; 536/27

According to International Patent Classification (IPC) or to both national classification and IPC

B. FIELDS SEARCHED

Minimum documentation searched (classification system followed by classification symbols)

U.S. : 424/93U; 514/44, 908; 536/27

Documentation searched other than minimum documentation to the extent that such documents are included in the fields searched

Electronic data base consulted during the international search (name of data base and, where practicable, search terms used)

APS, Chemical Abstracts

C. DOCUMENTS CONSIDERED TO BE RELEVANT

Category*	Citation of document, with indication, where appropriate, of the relevant passages	Relevant to claim No.
X	EMBO Journal, Volume 7, Number 4, issued 1988, P. Qiu et al. "Primary Structure of c-kit: Relationship with the CSF-1/PDGF Receptor Kinase Family - Oncogenic Activation of v-kit involves deletion of Extracellular Domain and C Terminus", pages 1003-1011, especially figure 2.	1-52
X	Cancer Research, Volume 51, issued 01 April 1991, T. Strohmeyer et al, "Expression of the hgt-1 and c-kit Protooncogenes in Human Testicular Germ Cell Tumors", pages 1811-1816, especially "Results" pages 1812-1815, entire document.	1-52
X	Journal of Immunology, Volume 140, Number 7, issued 01 April 1988, D. Harel-Bellan et al, "Specific Inhibition of c-myc Protein Biosynthesis using an Antisense Synthetic Deoxy-Oligonucleotide in Human T Lymphocytes", pages 2431-2435, entire document.	1-52



Further documents are listed in the continuation of Box C.



See patent family annex.

* Special categories of cited documents:	"T"	later document published after the international filing date or priority date and not in conflict with the application but cited to understand the principle or theory underlying the invention
"A" document defining the general state of the art which is not considered to be part of particular relevance	"X"	document of particular relevance; the claimed invention cannot be considered novel or cannot be considered to involve an inventive step when the document is taken alone
"E" earlier document published on or after the international filing date	"Y"	document of particular relevance; the claimed invention cannot be considered to involve an inventive step when the document is combined with one or more other such documents, such combination being obvious to a person skilled in the art
"L" document which may throw doubts on priority claim(s) or which is cited to establish the publication date of another citation or other special reason (as specified)	"A"	document member of the same patent family
"O" document referring to an oral disclosure, use, exhibition or other means		
"P" document published prior to the international filing date but later than the priority date claimed		

Date of the actual completion of the international search

11 August 1992

Date of mailing of the international search report

19 AUG 1992

Name and mailing address of the ISA/
Commissioner of Patents and Trademarks
Box PCT
Washington, D.C. 20231

Authorized officer

DEBORAH CROUCH, PH.D.

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Telephone No. (703) 308-1126